

AMENDMENTS TO THE CLAIMS:

Claim 57 is canceled without prejudice or disclaimer. Claims 56, 59 and 61-63 are amended. Claims 64-65 are added. The following is the status of the claims of the above-captioned application, as amended.

Claims 1-39 (Canceled)

Claim 40. (Withdrawn) A method for constructing a variant of a parent subtilase, wherein the variant has at least one altered property as compared to said parent subtilase, which method comprises:

- a) analyzing the three-dimensional structure of the parent subtilase to identify, on the basis of an evaluation of structural considerations in relation to a JP170 three dimensional structure, at least one amino acid residue or at least one structural region of the subtilase, which is of relevance for altering said property;
- b) modifying the DNA of the polynucleotide encoding the parent to construct a polynucleotide encoding a variant subtilase, which in comparison to the parent subtilase, has been modified by deletion, substitution or insertion of the amino acid residue or structural part identified in i) so as to alter said property;
- c) expressing the variant subtilase in a suitable host, and
- d) testing the resulting subtilase variant for said property.

Claim 41. (Withdrawn) A method of producing a subtilase variant, wherein the variant has at least one altered property as compared to a parent subtilase, which method comprises:

- a) producing a model structure of the parent subtilase on the three-dimensional structure of BPN', TY145 or JP170; or producing an actually determined three-dimensional structure of the parent subtilase,
- b) comparing the model or actual three-dimensional structure of the parent subtilase to the JP170 structure by superimposing the structures through matching the CA, CB, C, O, and N atoms of the active site residues,
- c) identifying on the basis of the comparison in step b) at least one structural part of the parent subtilase, wherein an alteration in said structural part is predicted to result in an altered property;

- d) modifying the nucleic acid sequence encoding the parent subtilase to produce a nucleic acid sequence encoding at least one deletion or substitution of one or more amino acids at a position corresponding to said structural part, or at least one insertion of one or more amino acid residues in positions corresponding to said structural part;
- e) performing steps c) and d) iteratively N times, where N is an integer with the value of one or more;
- f) preparing the variant resulting from steps a) - e);
- g) testing the properties of said variant; and
- h) optionally repeating steps a) - g) recursively; and
- i) selecting a subtilase variant having at least one altered property as compared to the parent subtilase.
- j) expressing the modified nucleic acid sequence in a host cell to produce the variant subtilase;
- k) isolating the produced subtilase variant;
- l) purifying the isolated subtilase variant and
- m) recovering the purified subtilase variant.

Claim 42. (Withdrawn) The method of claim 40, wherein the parent subtilase belongs to the sub-group I-S1 type subgroup, I-S2 type subgroup, TY145 type subgroup, or JP170 type subgroup.

Claim 43. (Withdrawn) The method of claim 42, wherein the parent JP170 type subtilase that is modelled is at least 58% homologous to SEQ ID NO:1, at least 60% homologous, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homologous to the sequence of SEQ ID NO:1.

Claim 44. (Withdrawn) The method according to claims 42, wherein the parent JP170 subtilase is a JP170 like subtilase which is at least 58% homologous to the sequence of SEQ ID NO:1, comprising the overall subtilisin fold and the following structural characteristics:

- a) a twisted beta-sheet with 7 strands,
- b) six alpha helices,
- c) at least three ion-binding sites,

and not comprising the Strong and Weak ion-binding sites of the BPN' like subtilases, wherein the positions of said three ion-binding sites in the three-dimensional structure of the subtilase is defined by the distance to the c-alpha atoms of the three active site amino acid residues of the subtilases, that is Ser, His and Asp, and the c-alpha atom of the amino acid residue next to the active site Ser residue (next to Ser), wherein said distances between:

- I) ion-binding site 1 and
 - i) Asp c-alpha atom is 26.70-28.70Å,
 - ii) His c-alpha atom is 22.10-24.10Å,
 - iii) Ser c-alpha atom is 16.95-18.95Å,
 - iv) next to Ser c-alpha atom is 15.30-17.30Å,
- II) ion-binding site 2 and
 - i) Asp c-alpha atom is 33.50-35.50Å,
 - ii) His c-alpha atom is 37-39Å,
 - iii) Ser c-alpha atom is 29.40-31.40Å,
 - iv) next to Ser c-alpha atom is 30.70-32.70Å,
- III) ion-binding site 3 and
 - i) Asp c-alpha atom is 41.50-43.50Å,
 - ii) His c-alpha atom is 42.90-44.90Å,
 - iii) Ser c-alpha atom is 34.50-36.50Å,
 - iv) next to Ser c-alpha atom is 35-37Å.

Claim 45. (Withdrawn) The method of claim 40, wherein the parent subtilase belongs to the JP170 type sub-group, and wherein the variant has at least one altered property as compared to a parent subtilase, which method comprises:

- a) producing a model structure of the parent JP170 type subtilase on the three-dimensional structure of JP170; or producing an actually determined three-dimensional structure of the parent subtilase,
- b) comparing the model or actual three-dimensional structure of the parent JP170 type subtilase to the BPN' or TY145 structure by superimposing the structures through matching the CA, CB, C, O, and N atoms of the active site residues,
- c) identifying on the basis of the comparison in step b) at least one structural part of the parent JP170 type subtilase, wherein an alteration in said structural part is predicted to result in an altered property;

- d) modifying the nucleic acid sequence encoding the parent JP170 type subtilase to produce a nucleic acid sequence encoding at least one deletion or substitution of one or more amino acids at a position corresponding to said structural part, or at least one insertion of one or more amino acid residues in positions corresponding to said structural part;
- e) performing steps c) and d) iteratively N times, where N is an integer with the value of one or more;
- f) preparing the JP170 type subtilase variant resulting from steps a) - e);
- g) testing the properties of said variant; and
- h) optionally repeating steps a) - g) recursively; and
- i) selecting a JP170 type subtilase variant having at least one altered property as compared to the parent subtilase.
- j) expressing the modified nucleic acid sequence in a host cell to produce the variant subtilase;
- k) isolating the produced JP170 type subtilase variant;
- l) purifying the isolated subtilase variant and
- m) recovering the purified subtilase variant.

Claim 46. (Withdrawn) The method of claim 45, wherein step (c) identifies amino acid residue positions located at a distance of 10Å or less to the ion-binding site of the JP170 type parent, preferably positions located at a distance of 6 Å or less.

Claim 47. (Withdrawn) The method of claim 45, wherein step (c) identifies amino acid residue positions in the JP170 type parent, the modification of which provides for the removal of the ion binding site by modification of at least one of the positions identified.

Claim 48. (Withdrawn) The method of claim 45, wherein step (c) identifies amino acid residue positions in highly mobile regions of the JP170 type parent.

Claim 49. (Withdrawn) The method of claim 45, wherein step (c) identifies amino acid residue positions in mobile regions of the JP170 type parent.

Claim 50. (Withdrawn) The method of claim 45, wherein step (c) identifies amino acid residue positions in the parent JP170 type, the modification of which may create at least one disulfide bridge by insertion of or substitution with at least one Cys residue.

Claim 51. (Withdrawn) The method of claim 45 wherein steps (c) and (d) provide for constructing a variant of a parent JP170 type having a modified surface charge distribution by:

- c') identifying, on the surface of the parent JP170 type, at least one charged amino acid residue;
- d') modifying the charged residue identified in step (a) through deletion or substitution with an uncharged amino acid residue;

Claim 52. (Withdrawn) The method of claim 45, wherein steps (c) and (d) provide for constructing a variant of a parent JP170 type having a modified surface charge distribution by:

- c'') identifying, on the surface of the parent JP170 type, at least one position being occupied by an uncharged amino acid residue;
- d'') modifying the charge in that position by substituting the uncharged amino acid residue with a charged amino acid residue or by insertion of a charged amino acid residue at the position.

Claim 53. (Withdrawn) The method of claim 45, wherein steps (c) and (d) provide for constructing a variant of a parent JP170 type having a modified surface charge distribution by:

- c''') identifying, on the surface of the parent JP170 type, at least one charged amino acid residue;
- d''') substituting the charged amino acid residue identified in step (a) with an amino acid residue having an opposite charge.

Claim 54. (Withdrawn) The method of claim 45, wherein step (c) identifies amino acid residue positions in the parent JP170 type, the modification of which to Pro may create a JP170 type variant exhibiting improved stability.

Claim 55. (Withdrawn) The method of claim 45, wherein step (c) identifies amino acid residue positions in the parent JP170 type at a distance of less than 10Å from the active site residues.

Claim 56. (Currently amended) A variant of a JP170 type subtilase comprising at least one modification in an amino acid residue in a ~~at least one~~ position located at a distance of 10Å or less to one or more ion binding sites, wherein the subtilase has at least 90% similarity to SEQ ID NO: 1 of ion-binding site 1, ion-binding site 2, or ion-binding site 3, or combinations thereof, wherein the at least one position of ion-binding site 1 is selected from the group consisting of 183, 184, 185, 186, 187, 188, 189, 191, 196, 197, 198, 199, 200, 201, 202, 203, 224, 225, and combinations thereof; wherein the at least one position of ion-binding site 2 is selected from the group consisting of 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, and combinations thereof; and wherein the at least one position of ion-binding site 3 is selected from the group consisting of 348, 350, 352, 363, 364, 365, 366, 367, 370, 394, 395, 396, 397, 398, 399, 400, 414, 415, 416, 417, 418, 419, 420, and combinations thereof; and wherein said positions correspond to the amino acid positions of SEQ ID NO:1.

Claims 57-58. (Canceled)

Claim 59. (Currently amended) A variant of a JP170 type subtilase comprising at least one modification in a mobile or highly mobile region, wherein the modification is in a position selected from the group consisting of:
 13, 14, 15, 16, 17, 18, 37, 38, 39, 40, 41, 42, 43, 47, 48, 49, 50, 58, 59, 60, 67, 96, 97, 98, 99,
~~407, 108, 109, 110, 111, 131, 132, 133, 134, 152, 153, 163, 164, 165, 166, 188, 189, 190, 191,~~
~~493, 495, 210, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 326, 327, 328, 329,~~
 330, 331, 332, 337, 338, 339, 340, ~~342, 355, 356, 357, 359, 360, 372, 373, 374, 375, 376, 377,~~
 378, 384, 385, 387, 388, 389, 390, 391, 392, 404, 405, 406, 407, 408, 409, 410, 411 and 419,
 wherein the subtilase has at least ~~90%~~95% similarity to SEQ ID NO: 1, and wherein said
positions correspond to the amino acid positions of SEQ ID NO:1.

Claim 60 (Previously presented) The variant of claim 56, wherein the at least one modification in an amino acid residue is in a position located at a distance of 6Å or less to the ion-binding site.

Claim 61 (Currently amended) The variant of claim ~~57~~56, wherein the at least one

modification comprises at least one of the modifications: ~~S193Q,Y; D196N; H200D, N; H200D, N+D196N; N390D; N391D; W392S,N,Q; and G394N,Q,F,Y,S and W392S,N,Q.~~

Claim 62 (Currently amended) A variant of a JP170 type subtilase having at least ~~80%~~95% similarity to SEQ ID NO: 1 comprising the introduction of an ion-binding site corresponding to the Strong ion-binding site of the BPN' like family subtilases, wherein said variant has a partial or full deletion of the region N79-N82 of SEQ ID NO:1 and subsequent insertion of one or more amino acid residues, and wherein said positions correspond to the amino acid positions of SEQ ID NO:1.

Claim 63 (Currently amended) The variant of claim ~~64~~62, wherein the sequence LNNSIQV (SEQ ID NO: 5) is inserted followed by the substitution A45D,N ~~and optionally the substitutions E44P,T and/or R47Q.~~

Claim 64 (New) The variant of claim 56, wherein the at least one modification comprises H200D+D196N or H200N+D196N.

Claim 65 (New) The variant of claim 63, further comprising at least one of the modifications: E44P,T and R47Q.